Author’s response to reviews

Title: Androgen-regulation of the protein tyrosine phosphatase PTPRR activates ERK1/2 signalling in prostate cancer cells

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Version: 7  Date: 29 December 2014

Author’s response to reviews: see over
Dear Cherry,

We are delighted that peer-review of our manuscript is now complete and that you feel our article is suitable for publication in *BMC Cancer*.

As requested, we have now added a complete list of author email addresses to the title page. We have also updated our methods section to include information on patient consent (page 15, lines 4-5), and revised our conclusions section according to the journal guidelines (page 10, line 23 onwards).

Once again we would like to thank the reviewers and your editorial team for the helpful comments received which we feel have improved the manuscript greatly. We trust our article is now suitable for publication and look forward to hearing from you shortly.

Yours Sincerely,

Jennifer Munkley

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Point by point response to reviewers:

Title: Androgen-regulation of the protein tyrosine phosphatase PTPRR activates ERK1/2 signalling in prostate cancer cells
Version: 4 Date: 14 October 2014
Reviewer: Michael Ladomery

Reviewer’s report:

Summary:
The role of androgens in driving prostate cancer is well established, but much still remains to be done in terms of understanding exactly how this occurs. This study starts with a genome-wide study aimed at identifying novel androgen-regulated genes. The study then wisely focuses on a single gene, PTPRR, which appears to be downregulated by androgen. Its regulation by AR is convincingly demonstrated in a range of appropriate PCa cell lines. PTPRR is also shown to be involved in the regulation of the RAS/ERK1/2 pathway - a highly significant finding.

Major Essential Revisions:
1. Ideally some transcription assays should have been included, for instance the dual luciferase/renilla assay using the PTPRR promoter, and with the putative AR binding sites mutated as well. I feel that this type of additional experiment would make it unequivocally clear that PTPRR is a direct (as opposed to indirect) AR target.

We agree with the reviewer that this would be an interesting experiment. A practical problem is the most promoter proximal AR binding site is 4.8kb from the gene (we have added information about ChIP-Seq binding site locations to Supplementary Figure 1). The PTPRR gene also contains a downstream intronic AR binding site and a far upstream site as well. Previous work has shown that AR binding sites within 100Kb of the gene can influence gene expression levels, although sites even further away could also be involved (1). Given the potential large distances involved to even the most promoter proximal site in terms of cloning, and the fact that AR association may also be indirect via a pioneer factor with another binding site we can’t predict (2), we thought the suggested dual luciferase/renilla assay using the PTPRR promoter as suggested would not give as clear results as hoped. However as the reviewer was concerned about whether PTPRR was a direct target of the AR, we carried out three new lines of additional experiments to investigate this further. These were examining the repression of PTPRR using a range of concentrations of R1881. We also show that androgen mediated repression of PTPRR is inhibited in the presence of the anti-androgen flutamide, and when the AR is depleted by esiRNA. This new data has been added to Figure 2 (sections C,F & G). In addition our time course data shows that PTPRR gene expression is repressed with 6 hours of treatment with R1881, and repression still occurs in the presence of the protein inhibitor cycloheximide, suggesting the repression is directly rather than indirectly mediated by the AR.

2. I am very persuaded by the cell line work - it is robust and several controls and derivative cell lines are used. What convinces me less is the analysis done on a very small patient cohort. N=7 is simply too small to be able to draw any conclusions. I totally appreciate the practicalities of looking at larger cohorts and the genetic heterogeneity and complexity of PCa cases, but as it stands Fig 4B really doesn't add enough to the paper. So it would be great if this aspect of the work could be enhanced significantly. Also pertinent to the focus of the paper, would it be possible to include some kind of AR status in the clinical samples. Alternatively, perhaps some mouse xenograft work could be carried out, eg orthotopic injection of cell lines in which PTPRR expression is manipulated.
We agree with the reviewer that it would be of value to examine PTPRR expression in a much larger patient cohort. We did have patient TMA available for use in this study, but unfortunately our antibody against PTPRR does not work on fixed tissue. So, in order to increase the impact of the paper, we have added data about PTPRR expression from publicly available datasets (shown in Figure 4B). Four out of six studies show a significant decrease in PTPRR expression in prostate adenocarcinoma, or comparing metastatic to primary adenocarcinoma (in two studies any changes were not significant). Our new text added to the results is:

'We examined PTPRR gene expression in prostate cancer clinical samples using previously published datasets that are publically available. Comparison of PTPRR expression by Affymetrix Array of 14 samples reported a 3.381 fold reduction in prostate cancer relative to normal tissue [17], and a 4.686 fold reduction in metastatic versus primary prostate cancer [18] (Figure 4B). PTPRR expression was also significantly reduced in 2 other previously published datasets [19-21] with 2 other datasets showing no significant changes in PTPRR gene expression [12, 22]' (page 8, line 10).

The six PCa samples used were all that we have available for this study. However, despite the small sample size we still feel that the information shown in Figure 4C will be of interest because it highlights that PTPRR expression is variable in clinical PCa samples, and so we would like to still include it for this reason. Based on the reviewers’ comments we have changed the wording of our discussion to acknowledge that the current dataset is small, and that further work will be required.

‘Further in vitro and in vivo studies are required to determine whether loss of PTPRR is a consistent event in PCa’ (page 10 line 19).

We have also added information about tumour grade and patient ethnicity to Figure 4C. Unfortunately information on AR status was not available for these patient samples used.

We also agree it would also be of extreme interest to carry out mouse xenograft work using cell lines in which PTPRR expression is manipulated, and thank the reviewer for this valuable suggestion. We have planned this for future work, since these experiments are beyond the scope of this study, and the time frame we were given for revisions to be addressed in a timely manner to allow the manuscript to be published with maximal impact.

Minor Essential Revisions:
1. A very minor point, but can the first results paragraph make it clear that the data presented in Figure 1 relates to LNCaP cells.
This has been added to the text (page 4, line 18).

2. In regards to Sup Fig 2, p.5 "binding sites were identified close to the PTPRR gene" - the word close is not very clear (how close?). Could you also illustrate the exact sequences that could be bound by AR; and provide a scale on this Figure.
The positions of the sequences bound by the AR are illustrated in supplementary Table 1. We have removed the phrase ‘close to the PTPRR gene’ and replaced with a reference to the exact position: ‘Three known AR binding sites were identified in the vicinity of the PTPRR gene, one of which was less than 5kb upstream, and another within an internal intronic region’ (page 5, line 1). A scale bar has been added to Supplementary Figure 1.

3. Fig3A, I note there is some baseline expression of PTPRR in the control. I wonder what might happen with siRNA mediated knockdown of PTPRR, might
pERK1/2 levels rise? this simple assay could lend additional weight to the conclusions.

Thank you. We have now carried out esiRNA mediated depletion of **PTPRR** in steroid deplete conditions, and added the result to Figure 3 (section D). Our findings indicate that depletion of **PTPRR** in these conditions did not have an effect on the levels of ERK1/2 phosphorylation. In the manuscript text we discuss that the impact of PTPRR on ERK1/2 in LNCaP cells is likely dependent also upon the other androgen-regulated modifiers of ERK1/2 signalling identified in Figure 1:

‘Although over-expression of PTPRR in androgen treated LNCaP cells was sufficient to repress ERK1/2 phosphorylation, depletion of PTPRR alone by esiRNA in steroid deplete LNCaP cells was insufficient to restore ERK1/2 phosphorylation (Figure 3D). This result suggests that additional androgen-regulated proteins are involved in modulating ERK1/2 phosphorylation in response to androgens. These additional genes likely include the other members of the RAS/ERK1/2 pathway identified by our IPA pathway analysis (Figure 1)’ (page 7, line 1).

4. Space permitting, I wonder whether a figure that illustrates PTPRR structure (gene and protein) might be of interest to the readership (many of whom are likely to be unfamiliar with it).

We agree that this would be of interest to the readership and have added this to Figure 2 (section I).

5. Could the discussion include (if known) information about normal tissue expression and developmental roles (if known) of PTPRR, and also if it is alternatively spliced. (On the latter point, I notice a 2004 study on murine Ptprr that describes several splice variants: PMID: 15461663).

Thank you, we had added information about this to the discussion section of the paper.

‘PTPRR is normally expressed in the brain, placenta, small intestine, stomach, uterus and weakly in the prostate. Mouse gene Ptprr encodes multiple protein tyrosine phosphatase receptor type R (PTPRR) isoforms, which display distinct patterns of expression during neural development, and negatively regulate mitogen-activated protein kinase (MAPK) signalling pathways; both ERK1 and ERK2 are hyperphosphorylated in the brains of mice deficient for PTPRR’ (page 9, line 18).

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interest

**References used in our response.**


Title: Androgen-regulation of the protein tyrosine phosphatase PTPRR activates ERK1/2 signalling in prostate cancer cells

Version: 4

Date: 7 October 2014

Reviewer: Lorna Harries

Reviewer's report:

Androgen-regulation of the protein tyrosine phosphatase PTPRR activates ERK1/2 signalling in prostate cancer cells. Munkley et al.

In this well written and interesting study, Munkley et al describe the androgen-regulation of the PTPRR gene, and provide evidence that decreased expression of this gene may be important in the progression of prostate cancer. This is an important finding from a well-executed study, and I have only a few comments.

Major compulsory revisions

1) Splicing patterns are commonly deregulated in prostate cancer. It would be interesting to see how many of the 226 novel androgen-regulated genes are alternatively spliced.

We agree that this would be very interesting and informative analysis. We analysed splicing in our previous study, and found that the validation rate for alternative events from our exon array analysis was only 23% (2), so we worry that any global interpretation of both differentially-regulated and alternatively-spliced genes would be unreliable. The reason for this is that exon arrays are not ideal for detecting splicing changes on a background of large gene expression changes (as occurs when cells are treated with androgens) due to the nature of probe technology (1). Future experiments using RNA-sequencing will hopefully shed more light on androgen-mediated splicing events that the reviewer suggests. We hope this is OK—we do appreciate the interesting suggestion of the reviewer.

2) How was multiple testing accounted for in the pathway analysis? Did the authors use a False Discovery Rate (FDR) adjusted q value, and if so what cut off was taken as representative of statistical significance?

The pathway analysis was done using Ingenuity Pathway Analysis (IPA) "Core Analysis" Function, which generates a score rather than a FDR. As the software used in the analysis is propriety we do not know how it accounts for multiple testing. We have added details of the networks analysed in Supplementary Table 1. The network shown in Figure 1 is number 18, with score 16.

3) I would like to see more details of the deregulated genes; what were the effect sizes, 95% CIs and the p/q values?

These data were published in our previous PLoS ONE paper (2), and so weren’t included in this manuscript. We have added p values to the graphs for the real-time PCR analysis performed on the androgen-regulated genes (Figure 1).

4) What are the characteristics of the LNCaP cells – how representative are they of primary prostate tissue?

The LNCaP cell line (ATCC® CRL-1740™) is a well-established androgen-dependent prostate cancer line (3, 4) which has been used extensively in the identification of androgen-regulated genes (5-8). The LNCaP line was established from a metastatic lesion of human prostatic adenocarcinoma from a 50-year-old caucasian male. However, like primary prostate tissue, the LNCaP cell line expresses a transcriptionally-active androgen receptor.
5) How were the doses and incubation times of androgen treatment determined? Are these levels physiologically relevant?

For most of the experiments in the paper we used 10nM of R1881. This was the same concentration as was used in our published exon array paper (2). Our previous work has shown that this concentration is optimal to detect gene expression changes. However, as this is higher than physiological levels of androgens (9), we have now treated LNCaP cells with a range of concentrations of R1881 and demonstrated that we still see repression across a range of concentrations. This data has been added to Figure 2 section C.

6) Why was PTPRR alone followed up functionally? What was the rationale for choosing this particular gene above the others identified from the pathway analysis for functional follow up?

Our pathway analysis and subsequent real-time PCR validation identified 7 novel androgen-regulated genes associated with ERK1/2 signalling. Of particular interest was SPRY1, however this gene has already been studied extensively in prostate cancer. As the PTPRR gene already had links to cervical and colorectal cancer we decided this would be an interesting target to study in prostate cancer. Although less is known about the other 5 genes, they would also be interesting to study in the future.

7) Please can the authors give more details about the donors for the primary prostate samples in terms of age, ethnicity, tumour grade, drug treatment etc? Do these samples have heterogeneous patterns of PTPRR expression because the samples are dissimilar?

Information regarding ethnicity and tumour grade has been added to Figure 4B. Information on treatment history was not available for these samples.

8) I am unsurprised that no differences are noted with HEK293 cells – these are embryonic kidney cells? Do the authors plan to look at other prostate cell lines?

The HEK293 cells used in this study are embryonic kidney cells which are PTEN positive as confirmed by western blot. We agree that it would be interesting to explore the effect of PTPRR in a range of prostate cancer cell lines of differing PTEN status and plan to do this in future work.

9) How were the endogenous controls chosen? Were they shown empirically to be unresponsive to androgens? Did the authors check amplifications for specificity given that SYBR green will bind any dsDNA?

For protein loading actin was used as an loading control. Actin has been used in numerous studies for this purpose (e.g. (8)). Work in our lab comparing expression of actin, GAPDH and B-tubulin protein levels in LNCaP samples (grown with or without androgens) has shown no difference between the three proteins, and confirms that to the best of our knowledge these genes are unresponsive to androgens. For real time PCR samples were normalised to actin, GAPDH and B-tubulin. We found that when equal amounts of RNA were used for protein synthesis we repeatedly obtained very similar ct values (within 1 ct for LNCaP cells grown with and without androgens), again indicating that these genes are not androgen responsive. Dissociation curves were analysed for all amplifications to ensure primer specificity.
Minor essential revisions

10) Minor point – there are a couple of places in the text where the symbols have become corrupted.
Thank you. This has been corrected. We think this was occurred during the conversion of our manuscript text document to pdf.

11) Minor point – please clarify on the graph axes precisely what is being measured – ie ‘PTPRR expression’ rather than ‘relative expression’
This has been changed on all of the graphs. Thank you.

12) Page 6 line 15, please italicise ‘Spry2’ if referring to the mouse gene.
This has been changed. Thank you.

References used in our response